ORIGINAL ARTICLE

Longitudinal Measurement of Hemodynamic Changes within the Posterior Optic Nerve Head in Rodent Nonarteritic Anterior Ischemic Optic Neuropathy

Jin Ma^{†1}, Ting Chen^{†1,2}, Yiwei Wang¹, Chan Zhao¹, Donghui Li¹,
Meng Wang¹, Linyang Gan¹, Yong Zhong^{1*}

¹Department of Ophthalmology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, China ²Department of Ophthalmology, Renmin Hospital of Wuhan University, Wuhan, 430060 China

Key words: nonarteritic anterior ischemic optic neuropathy; rat model; posterior optic nerve head; blood flow; *in vivo*

Objective To assess the *in vivo* dynamic blood flow features of posterior optic nerve head (ONH) in rat model of nonarteritic anterior ischemic optic neuropathy (rNAION).

Methods rNAION was established with Rose Bengal and argon green laser in Sprague-Dawley rats. Fundus photography and fundus fluorescein angiography (FFA) were performed to assess the dynamic changes of optic disc in morphology in 90 days and in blood perfusion in 3 hours after the induction of disease. Histological examinations were performed to evaluate the success of modeling. The dynamic blood flow kinetics of posterior ONH in rNAION were measured by Laser Doppler Flowmetry (LDF) on the day 3, 7, 14, 21, and 40 after the disease induction. One-way ANOVA, Student's *t*-test and Bonferroni adjustment were used for multiple comparisons of kinetic measurements of blood flow.

Results Optic disc edema and subsequent resolution associated with the development of optic disc pallor were observed in rNAION. FFA showed that the optic disc was hypofluorescence in the early phase and hyperfluorescence in the late phase. Histological studies suggested edema and loosened tissues of ONH, loss of retinal ganglion cells (RGCs), optic nerve substance and gliosis. Compared to the naive rats, the blood flow kinetics of posterior ONH in rNAION significant reduced at each time point after modeling ($F=175.06$,

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*Corresponding author Tel: 86-10-69156351; E-mail: yzhong_eye @163.com

†Jin Ma and Ting Chen contributed equally to this work.

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$P < 0.0001$). The reductions were specifically remarkable in 14 days after the disease induction (All $P < 0.01$).

Conclusions Continuous blood perfusion reduction was found in rNAION, with significant alteration in 14 days after disease induction. Our results provided important information for understanding the hemodynamic changes in rNAION.

NONARTERITIC anterior ischemic optic neuropathy (NAION) is one of the major causes of acute optic neuropathy in the elderly.^{1,2} Despite the wide consensus on the "ischemic" nature and the gross anatomical location of this disease as the name implies, the exact underlying hemodynamic mechanism within the optic nerve head (ONH) remain to be fully elucidated.

The ONH is vascularized by two major arterial systems:³ the superficial nerve fiber layer is mainly supplied by retinal arterioles; the deep major part of the ONH, subdivided into prelaminar, lamina and retrolaminar regions, are principally supplied by direct branches of the posterior ciliary arteries (PCAs). It has been suggested that it is the deep ONH regions, supplied by PCAs, that probably show more decrease in circulation⁴ and are involved in the pathogenetic changes in NAION.⁵⁻⁷ However, most current available techniques for blood flow measurement in NAION patients, such as Laser Doppler Flowmetry (LDF), laser speckle flowgraphy, can only measure the blood flow of superficial nerve fiber layer in NAION patients.^{3,8} LDF is one of the non-invasive techniques for assessment of volumetric blood flow.⁹ It has been widely used to measure the hemodynamics of cerebral vascular after stroke,^{10,11} and also in the measurement of blood flow in the optic nerve head¹² and subfoveal choroid.¹³ Rodents are significant animal model in physiology and therapeutics, and similar LDF technique has been developed to measure the vascular bed of the rat eyes.¹⁴

A rat NAION (rNAION) model with similar fundoscopic and pathological changes to those of human NAION was first established by Bernstein in 2003.^{15,16} The key event to generate this model is to induce photochemical thrombosis of the microvasculature in the ONH by direct irradiating the ONH with argon green laser after intravenous injection of Rose Bengal (RB), which results in ischemic ONH edema.¹⁷ With the rNAION model, some studies have observed the deeper ONH circulation alterations *in vitro* ONH tissues.^{15,18,19} The strength of these results, however, is limited by the "*in vitro*" nature of the experimental approaches.

In addition, the microcirculation status of ONH was only measured at the very early phase (no more than 3 days) after disease induction, which only provided a snapshot of the dynamic process and may obscure the speed and duration of arterial blood flow reduction in ONH during disease progression.

In our previous studies, the structure and function changes have been longitudinally monitored.^{15,19} To shed new light on the characteristics of the hemodynamic changes in rNAION at different time points, dynamic changes in blood flow kinetics of the ONH were measured "*in vivo*".

MATERIALS AND METHODS

Animals

All protocols were approved by the Experimental Animals Committee and were consistent with the Declaration of Helsinki or the NIH statement for the Use of Animals in Ophthalmic and Vision Research. Male pathogen-free Sprague-Dawley (SD) rats (weighing 200-220 g, kept at 20-22 °C) were used for this study. Each rat in this study underwent a complete ophthalmic examination to rule out ocular abnormalities.

Induction of rNAION

The experimental protocol for rNAION induction has been reported previously.^{15,19} In brief, 1 ml/kg Rose Bengal (RB) [2.5 mM in phosphate-buffered saline (PBS), 90% purity; Sigma-Aldrich, St. Louis, MO, USA] was injected via femoral vein and the right ONHs were immediately photo-activated using an argon-laser photo-coagulator (Ultima 2000 SE Argon, Coherent inc., Santa Clara, CA, USA) with the following parameters: wave length 532 nm; power 50 mW; laser spot size 500 μ m; duration 12 seconds. The posterior segments of the eyes were examined immediately after induction to rule out hemorrhage. For the laser group, the ONH of the right eyes were illuminated with the laser without intraperitoneal injection of RB; and vice versa for the RB group (only injected with RB via the femoral vein). The naive group received no intervention as the

name indicates.

Fundus photography and fundus fluorescein angiography

Fundus photography and fundus fluorescein angiography (FFA) were performed after anesthetization and mydriasis using a slit lamp equipped with a camera (ASA 800, incandescent; Nikon, Tokyo, Japan) and Spectralis HRA+OCT (Heidelberg Engineering, Heidelberg, Germany), respectively. For FFA, angiographic images centered on the ONH were taken at intervals of approximately 1 second immediately after injection of fluorescein sodium for about 20 seconds, and continued at intervals of about 10 seconds for up to 5-9 minutes. Fundus photographs were taken in naive rats ($n=2$) and rNAION 3 h, 1 d, 5 d, 14 d and 90 d after disease induction ($n=3$ for each time point). FFAs were performed for naive rats ($n=2$) and rNAION ($n=3$) in 3 hours after disease induction. The early phase of FFA was defined as before 1.5 minutes after injection, and late phase was defined as over 3 minutes after injection.

Histologic preparation

Animals were euthanized after deep anesthetization, and the eyes were removed immediately and then fixed in 4% paraformaldehyde-phosphate-buffered saline (PF-PBS) for 1 h. Tissues were then embedded in paraffin, sectioned, and stained with hematoxylin-eosin (H & E) or toluidine blue for histologic examination. Histologic examinations were performed in naive rats ($n=2$), laser-only rats and RB-only rats (1 d after treatment, $n=2$ for each group), and rNAION rats 1 d and 90 d after disease induction ($n=2$ at each time point).

Laser doppler flowmetry

Longitudinal hemodynamic changes of posterior ONH were monitored with laser doppler flowmetry (LDF) device (moorVMS-LDF2, Moor Instruments Ltd., UK) on day 3, day 7, day 14, day 21 and day 40 after disease induction in rNAION rats ($n=3$ at each time point) and in naive rats. The surface laser Doppler probe (VP3.0, Moor Instruments Ltd., UK), with a tip diameter of 0.50 mm, operated at the wavelength of 785 nm. As previously described,²⁰ the optic nerve of rat was exposed through a lateral canthus incision. The LDF probe was placed perpendicular to the surface of the optic nerve and as close as possible to the eyeball. The volume of LDF measurement was approximately

1 mm³. The ONH blood flow kinetics, measured in perfusion units (PU) on a real-time basis, was monitored for about 3 minutes till the readout reached steady state. PU is the basic index of LDF measurement and is the Doppler shift value produced by RBC flowing. It is a relative unit to measure the microcirculatory blood flow in local deep tissue and the change of PU value directly shows the changes of microcirculatory blood flow of tissue.

Statistical analysis

All data were reported as mean $\pm$ SD. Statistical analysis was performed using SAS (version 9.3, SAS Institute Inc, Cary, NC) to evaluate longitudinal changes in ONH blood perfusion over time. Statistical differences were evaluated by one-way ANOVA, Student's *t*-test and Bonferroni adjustment for multiple comparisons. A two-sided *P* value less than 0.05 was considered statistically significant.

RESULTS

Fundus photography and FFA of rNAION

Fundus photography (**Fig. 1**) showed that, in contrast to fundus of naive eyes, the edema of ONH in the rNAION eyes was observed at 3 hour after disease induction and reached maximum on day 1 after disease induction. On day 5, profound resolution of ONH edema was observed in the rNAION eyes, and the appearance of ONH returned to normal on day 14; on day 90, however, the ONH appeared to be pale and reduction in size.

FFA (**Fig. 2**) revealed the normal fluorescein perfusion of the choroidal and retinal vasculature in the early phase and normal fluorescein distribution of fundus in the late phase in naive rats ($n=2$). In rNAION rats 3 hours after disease induction, however, filling defects in the choroid and ONH were observed in the early phase, and marked dye leakage from the ONH was present in the late phase.

Histopathology

As compared with naive eyes, no abnormal changes were observed in the retina and ONH in the laser-only rats 1 day after laser treatment and RB-only rats 1 day after RB injection. In the rNAION rats, however, obvious ONH edema, usually associated with peripapillary retinal detachment, was observed on the day 1 after disease induction; and on the day 90, a reduction in

Figure 1. Fundus photographs of optic nerve head (ONH) in naive rats and rNAION rats. A. The border of the ONH in a naive rat eye was distinct (arrow); B. in rNAION rats, blurring of the ONH border (arrow) observed 3 hours after disease induction, indicating edema; C. diameter of the ONH enlarged and reached the maximum (arrow) 1 day after disease induction; D. five days after induction, edema of the ONH resolved almost completely; E. the appearance of the ONH returned to normal on day 14; F. the ONH was apparently pale with reduction in size on day 90. rNAION: rat model of nonarteritic anterior ischemic optic neuropathy; ONH: optic nerve head.

Figure 2. Representative FFA images of a naive eye and an rNAION eye 3 hours after disease induction. A. normal fluorescein perfusion of the choroidal and retinal vasculature in the early phase of FFA in a naive eye. B. normal fluorescein distribution in the fundus in the late phase of FFA in a naive eye; C. FFA imaging 3 seconds after injection (early phase) of an rNAION eye showed filling defects in the choroid and the ONH (arrow); D. late phase imaging of the same eye showed marked dye leakage (arrow) from the ONH. FFA: fundus fluorescein angiography.

number of retinal ganglion cell (RGC) axons, as well as tissue fibrosis and cellular infiltration were observed, while cell densities of inner nuclear layer (INL) and outer nuclear layer (ONL) remained unchanged (**Fig. 3**).

Myelinated axon bundles of retinal ganglion cells (RGCs) were tightly packed and surrounded by thin pial septate in the optic nerve (ON) in naive rat eyes. On the day 90 after rNAION induction, however, there was a marked loss of central axons with increased septal thickness and shrinkage of the axonal bundles (**Fig. 4**).

Longitudinal measurement in hemodynamic changes of posterior ONH blood flow in rNAION

The mean blood flow kinetics of naive eyes was 174.73 ± 5.34 PU ($n=3$), and the mean blood flow of rNAION eyes on day 3, 7, 14, 21 and 40 after NAION modeling were 116.12 ± 8.93 PU ($n=3$), 94.89 ± 1.83 PU ($n=3$), 73.02 ± 6.99 PU ($n=3$), 68.94 ± 2.24 PU ($n=3$) and 60.92 ± 4.58 PU ($n=3$) respectively, where a significant difference was noticed ($F=175.06$, $P<0.0001$) with a rapid and pronounced drop of blood perfusion in posterior ONH observed (**Fig. 5**). The reduction in blood perfusion was statistically significant at each time point after disease induction as compared with that in naive rats (all $P<0.0001$). The blood perfusion significantly decreased compared with that in naive rats, with a 33.55%, 45.69% and 58.21% reduction on day 3, day

Figure 3. Representative histologic sections of ONH and retina in naive and rNAION eyes (H&E stain). A. longitudinal sections of the ONH in naive eyes, B. in the laser-only eyes and C. in the RB-only eyes showed normal histology and anatomy of the optic nerve and peripapillary retina. D. On day 1 after rNAION induction, the ONH was edematous with thickened nerve fiber bundles (double asterisks) and peripapillary retinal detachment (arrow). E. On day 90 after induction, there was a reduced number of RGC axons (long arrow) accompanied by gliosis and cellular infiltration (short arrow). F. naive eyes, G. laser-only eyes, H. RB-only eyes, and I. eyes 1 day after rNAION induction. The RGCs in peripapillary retina were closely packed in a single layer with normal density. J. On day 90 after rNAION induction, there was an obvious reduction in density of the RGCs in retina, while the cell densities in the INL and ONL remained mainly unchanged. (magnifying power: A-E, 50 $\times$; F-J, 200 $\times$). RB: rose bengal; RGC: retinal ganglion cell; INL: inner nuclear layer; ONL: outer nuclear layer.

Figure 4. Representative cross sections of ON in naive and rNAION eyes (Toluidine blue stain, 200 $\times$). A. cross section of ON in naive eye showed tightly packed axonal bundles by the pial septate (S); B. cross section of ON on day 90 after rNAION induction, an apparent reduction in central axonal bundle density with increased septal thickness was observed. AxB: axonal bundle. ON: optic nerve.

7 and day 14, respectively. The differences were significant between the data of day 3 and day 7 ($t=4.66$, $P=0.008$), day 7 and day 14 ($t=4.81$, $P=0.006$), day 3 and day 14 ($t=9.47$, $P<0.0001$). No significant differences demonstrated between neither day 14 and day 21 ($t=0.90$, $P=1.00$), nor day 21 and day 40 ($t=1.76$, $P=1.00$).

DISCUSSION

Nowadays, plenty of experimental researches using rNAION have been conducted to investigate cellular inflammation,²¹ oligodendrocyte death²² and neuro-

protective effects of drugs.^{23,24} The blood flow of ONH has been observed *in vitro* in rNAION. Bernstein¹⁵ used intra-cardiac perfusion of India ink and found a severe reduction in the filling of ONH capillaries 30 minutes after induction. Chuman *et al.* also found the reduction of capillary perfusion in the ONH 3 days after induction by using the same method.¹⁹ However, Nicholson *et al.* used perfusion of fluorescent marker (FITC-BSA) and showed a quite different kinetic of microcirculation impairment in the posterior segment of ONH with detectable but minimal loss of capillary perfusion 4 hours after disease induction and a much more significant loss of perfusion 1 day after induction.¹⁸ It is worth

Figure 5. The hemodynamic changes in blood flow kinetics of posterior ONH in naive rats and rNAION rats at different time points after disease induction. The posterior ONH blood perfusion measurements in rNAION rats were significantly lower than that in naive rats (all $P < 0.0001$). Statistically significant reductions of optic nerve blood flow were detected between day 3 and day 7, day 7 and day 14 after disease induction (both $P < 0.01$).

noting that in these studies, microcirculation status of the ONH was only measured at the very early time after disease induction.

In this study, we produced the rNAION following the method of Bernstein.⁹ Optic disc edema appeared as a hallmark in the early phase of disease and the FFA revealed early hypofluorescence followed by late hyperfluorescence in the optic disc. Histopathological study showed tissue edema followed by loss of axonal bundles, thickening of septa and gliosis in the optic nerve. These results demonstrated that there was a selective damage to capillaries in ONH, followed by ONH edema, and finally optic atrophy with loss of RGC. Therefore, we believed that the rNAION in our study was successfully established as Bernstein did.

Then, we longitudinally measured the dynamics of blood flow kinetic changes in ONH of rNAION by placing LDF probe directly 1 mm anterior to the optic nerve (the posterior ONH), where the most prominent degenerative changes were detected histologically.²⁵ Interestingly, in rNAION, the ONH blood perfusion did not reduce uniformly in depth. A sharp reduction of blood perfusion was found in 14 days after the disease induction, while in the longitudinal observation of the posterior ONH blood flow, the blood perfusion remained low steadily afterwards, indicating that the ischemic status was then stable. The underlying mechanism may lie in that in the early time, the capillary is blocked by fibrin, capillary leakage and extracellular edema result in intracellular edema and/or capillary compression,¹⁸ while in the late time, the impaired blood flow autoregu-

lated^{25,26} after photochemical thrombosis of microvasculature in the ONH.

In rNAION, we found that as the progress of the disease, even when the edema of the ONH had resolved, the density of RGCs still reduced. Our finding was consistent with the report in 2016.²⁷ The coupling association between blood flow and neural activity in neural tissues has been demonstrated in literatures.²⁸ Relationship had been also found between ischemic status and deteriorated visual function, damaged structure and other morphometric changes.^{29,30} Thus we hypothesized that the reduced blood flow might be the reflection of the reduced metabolic demand associated with neuronal loss and connective tissue changes.

Despite we firstly observed *in vivo* the dynamic feature of posterior ONH blood flow in rNAION in this study, limitations of this study should be addressed. Firstly, the approach used to measure the posterior ONH blood flow was invasive and the surgical exposure of the posterior ONH was of high skilled operation. Secondly, we did not measure RGCs density of the retina which might be associated with the blood flow of posterior ONH, thus we were not able to provide a quantitative assessment of the neurodegeneration. Thirdly, functional evaluations of the ON, e.g. visual evoked potential, were not carried out in this study. These limitations should be kept in mind when interpreting the data and should be taken into consideration for studies in future.

In summary, we successfully established rNAION. With this model, our study characterized the *in vivo* dynamic feature of posterior ONH blood perfusion reduction at different time points. Our findings, to some extent, provide a reference for understanding the hemodynamic changes occurred in rNAION. Further studies on ameliorating the early blood flow with rNAION will provide valuable information on the therapeutic approaches.

Conflict of interest statement

All authors declared no conflict of interests.

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